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| (54) Title: FURTHER IMPROVEMENTS RELATING TO DRUG DELIVERY SYSTEMS (57) Abstract <p>A three component system, for use in association with one another, in the treatment of malignant diseases comprises: a first component which is a substance or conjugation of substances characterised by (a) one or more molecular configurations that are complementary to molecular configurations associated with malignant cells, such that the first component tends to localise selectively at sites of malignant cells and (b) additionally by one or more catalytic sites; a second component which is able to bind to such part of the first component so as to inactivate the catalytic site of the first component and/or accelerates the clearance of the first component from the blood when the first and second components are administered clinically and a third component which is a substrate for the catalytic site on the first component, one of the end products of the catalysis being a substance which is more cytotoxic to malignant cells than the third component. The use of the second component reduces the extent to which the cytotoxic drug is released from the third component in regions of a patients body remote from the target tumour.</p> | | |

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TITLE: FURTHER IMPROVEMENTS RELATING TO DRUG
DELIVERY SYSTEMS

THIS INVENTION relates to methods and systems for the control of neoplastic cell growth and is particularly concerned with methods and systems involving the localisation of cytotoxic agents at tumour sites.

5 In our earlier Patent Application PCT/GB88/00181 we disclose a two-component system which comprises

(i) a first component (Component A-E) that is an antibody fragment capable of binding with a tumour
10 associated antigen, the antibody fragment being bound to an enzyme capable of converting a prodrug into a cytotoxic drug;

(ii) a second or final component (Component PD) that is a prodrug convertible under the influence of the enzyme
15 to a cytotoxic drug (CD).

In our earlier PCT Patent Application and in this present Patent Application, the word 'tumour' is to be understood as referring to all forms of neoplastic cell growth including carcinomas, sarcomas, lymphomas and leukaemias.

20 Our existing system is used to target cytotoxic prodrugs to the site of neoplastic cell growth. However, although this two-component system is useful in the control of tumours, the amount of first component that localises per gram of tumour in humans may be less than

0.1% the total amount of first component administered. A substantial proportion of the non-localised first component including active enzyme remains in the circulating blood. It is therefore desirable to reduce the amount of this freely circulating antibody-enzyme first component before administration of the prodrug so as to limit the formation of active drug in blood from where it would be carried to normal tissues. A similar problem arises when the first component is prepared from whole antibody.

The present invention provides methods for the removal from blood of residual conjugates of the enzyme or for inactivation of the enzyme in the residual first component with whole antibody or antibody fragment or equivalent component after the first component has localised at tumour sites.

The present invention is concerned with reducing problems arising in the clinical use of our above-mentioned two-component system from the presence in the patient of non-localised first component and permits further extension to be made to the nature of the first component.

The present invention provides a three-component system, for use in association with one another, in the treatment of malignant diseases comprising: a first

component which is a substance or conjugation of substances characterised by (a) one or more molecular configurations that are complementary to molecular configurations associated with malignant cells, such that the first component tends to localise selectively at sites of malignant cells and (b) additionally by one or more catalytic sites; a second component which is able to bind to such part of the first component so as to inactivate the catalytic site of the first component and/or accelerates the clearance of the first component from the blood when the first and second components are administered clinically; a third component which is a substrate for the catalytic site on the first component, one of the end products of the catalysis being a substance which is more cytotoxic to malignant cells than the third component.

The clinically most useful form of the first component is a conjugate of an antibody or fragment thereof and an enzyme while the clinically most useful form of the third component is a prodrug convertible under the influence of the enzyme activity of the first component into a cytotoxic compound. The antibody will desirably be one recognising and binding preferentially to a tumour associated antigen and it will be apparent to those skilled in the art how to match the antigen

associated with the particular tumour to be treated with the antibody or fragment to be used in the first component, how to match the cytotoxic compound to the tumour to be treated and how to match the prodrug to the enzyme activity of the first component.

As described in our above-mentioned copending International Patent Application PCT/GB88/00181 the prodrug can be benzoic acid mustard glutamide that converts to benzoic acid mustard [p-(bis-2-chloroethyl)amino]benzoic acid under the influence of a carboxypeptidase. However, the principles of this invention are equally applicable to other prodrugs releasing benzoic acid mustard or analogues thereof or other cytotoxic drugs using enzymes appropriate to the removal from the prodrug of the structural feature distinguishing the prodrug from the cytotoxic drug.

When antibody is used in the first component, it can be whole antibody or one of the antibody fragments, e.g. $F(ab')_2$ or other fragment as described in our above-mentioned earlier filed International Patent Application. The function of the antibody in the first component is to assist in the localisation of the first component in the region of the tumour to be treated and this function can also be fulfilled by substances other than antibodies, e.g. hormones or growth factors that have

affinity to other tumour-associated compounds.

In one embodiment of the system of the invention the first component is a conjugate of an antibody to a tumour-associated antigen or a fragment thereof that includes the antigen binding site of the antibody, said antibody or fragment thereof being conjugated directly, or indirectly through a linking component, to an enzyme or to an antibody or antibody fragment with catalytic functions. In this case the conjugation can be effected by chemical bonding or by splicing together nucleic acid sequences that code at least for one or more antigen binding sites and one or more catalytic sites and such other sequences as are necessary to retain the vector function of the molecule and the catalytic function of the peptide when the gene product of the reconstructed nucleic acid sequence is expressed by eukaryotic or prokaryotic cells.

In a further embodiment, the antibody in the first component is bivalent and formed by bonding together two univalent antibody fragments, or by recombinant DNA techniques, one fragment having affinity for a tumour marker substance, the other having affinity for an enzyme. In such a case the conjugate can be formed either in vitro prior to administration or in vivo by first administering the bivalent antibody, allowing time for it to localise at tumour sites and then administering the enzyme for

capture by the second arm of the antibody localised at tumour sites.

The antibody of the first component may be a human immunoglobulin, or fragment thereof, having antigen binding site(s) of human origin or having antigen binding site(s) of non-human species.

Reichmann L, Clark M, Waldmann H, and Winter G (Reshaping human antibodies for therapy - Nature 332: 323-327, 1988) shows that by genetic engineering techniques the antigen binding sites of a rodent monoclonal can be incorporated into human immunoglobulin fragments so that the immunogenicity of the molecule in the human subject is minimised. It has been shown that immunoglobulin-gene DNA can be manipulated so that the Fc portion of the antibody has been replaced with an active enzyme moiety (Neuberger MS, Williams GT, Fox RO - Nature 312: 604-608, 1984) and such genetically engineered constructs bearing one or more antigen binding sites and one or more enzyme active sites can be used in the present invention.

It has been observed that when monoclonal antibodies derived from one species are injected into another species the host antibody response may be (at least partially) directed at the idiotype of the injected monoclonal. (Rowe et al, IRCS Med Sci. 13: 936-7, 1985). Similarly, it is well-known that bacterial products,

including enzymes, are immunogenic in mammalian species including man.

The present system will be most effective in man and suitable for repetitive use when the immunogenicity of a first component antibody-enzyme conjugate is minimised or if immune tolerance to such conjugates has been induced. This is likely to be achieved through genetic engineering methods since the production of monoclonals to specific antigens by human hybridomas has so far proved difficult to achieve consistently. It has been shown that the antigen binding site of a rodent monoclonal antibody can be incorporated into a human immunoglobulin framework (Reichmann et al, Nature 332: 323-327, 1988).

It has also been shown that antibodies can be produced which function as enzymes (Pollack SJ et al, Science 234, 1570-1573, 1986) so the ultimate form of the antibody-enzyme conjugate may be a human immunoglobulin construct expressing one or more antigen binding sites characterised by peptide sequences of non-human origin and one or more catalytic sites.

With a 'humanised' antibody conjugated to a human enzyme or a non-human enzyme which has been rendered non-immunogenic in man, or a construct with both antigen binding and catalytic sites on a human immunoglobulin, the second component of our system will need to be directed at

either the active site of the enzyme or at the idiootype of the antibody since a clearing antibody against the generality of human immunoglobulins would be unsuitable.

We have developed several different methods of removing what becomes the unwanted circulating first component after maximum localisation of the first component has occurred in the region of the tumour to be treated. The exact nature of the second component will depend upon the particular strategy to be used for removal of non-localised first component but the second component will always be one that either inactivates the catalytic site in the first component and/or accelerates its clearance from the blood.

According to one embodiment the second component is an antibody or fragment thereof having an affinity for an antigen binding site of an antibody of the first component or the active site of an enzyme of the first component or another constituent part of the first component.

According to a further embodiment the second component is one causing rapid loss of enzyme activity of the first component in plasma without incurring significant loss of enzyme activity from tumour sites.

According to a further embodiment the second component includes a sufficient number of covalently bound

galactose residues or residues of other sugars such as lactose or mannose, so that it can bind enzyme in plasma but be removed together with the enzyme or antibody-enzyme conjugate from plasma by receptors for galactose or other
5 sugars in the liver in a period of time such that the antibody does not, to any appreciable extent, enter the extravascular space of the tumour where it could inactivate tumour localised enzyme. In this case,
10 galactose residues in the second component are either chemically added or exposed by removing terminal sialic acid residues.

Terminal sialic residues play a role in maintaining the presence of glycoproteins in the blood.
15 Removal of terminal sialic acid by neuraminidase exposes proximal sugar residues such as galactose. Desialylated proteins are rapidly removed from the blood by receptors in liver and possibly other sites. (Morell et al, J.Biol. Chem. 246: 1461-1467, 1971).

20 Asialo human chorionic gonadotrophin was prepared by digesting 1 mg of a glycoprotein in 1 ml of 0.05M sodium acetate buffer, pH 5.6, containing 0.15M NaCl with 20ug of neuraminidase (Sigma Type II from Vibrio cholerae) at 37° for 30 minutes. The neuraminidase was
25 then removed. Sialo and Asialo preparations were compared for clearance in A2G mice. T 1/2 for the sialyted hCG was

in excess of 24 hours but $T_{1/2}$ for the desialyted form was <5 min.

A further embodiment of the invention is one wherein the first component is an antibody enzyme
5 conjugate modified by addition of, or exposure of, additional galactose or other sugar residues, and is for administration in conjunction with an agent, such as asialofetuin, which has greater affinity for the corresponding sugar receptors which are involved in
10 removing galactosylated or similar proteins from the blood, the asialofetuin blockade being maintained until satisfactory levels of conjugate have been attained in the tumour, then allowing the concentration of the conjugate in the plasma to fall before administering the third
15 component.

This embodiment requires the addition of galactose residues to the antibody-enzyme conjugate by methods similar to those described for galactosylating the clearing antibody. Before administering the
20 galactosylated conjugate the galactose receptors are blocked by an agent which binds more avidly to those receptors than the galactosylated conjugate. This results in maintenance of a high level of conjugate in the plasma until galactose receptors are again free to take up the
25 conjugate.

Asialofetuin is a substance known to bind strongly to galactose receptors but other less immunogenic

substances could be identified or developed for the same purpose.

A further embodiment of the invention is one wherein the second component is conjugated to a
5 macromolecule such as a dextran, liposome, albumin microsphere or macroglobulin with a molecular weight in excess of 500,000 Daltons or a biodegradable particle such as a blood group 0 erythrocyte so that the second component is restrained from leaving the vascular
10 compartment by virtue of the size of the conjugate.

A further embodiment of the invention is one wherein the second component is an antigen, hapten or protein construct bearing an epitope capable of binding with the first component to form complexes having
15 accelerated clearance from plasma.

In a still further modification of the system, the first component is covalently linked to biotin or derivatives of biotin, and the second component then comprises the biotin-binding glycoprotein avidin found in
20 egg white, or streptavidin, itself optionally covalently linked to galactose.

Biotin may be conjugated to antibody or fragment thereof by reaction with a 10 molar excess of sulphosuccinimidyl 5-(biotinamido)hexanoate at pH 8.5 at
25 4°C for 16 hours. The product is purified by

chromatography on Sephadex G-25.

In all these cases, except for the procedure involving asialofetuin, the second component may be conjugated with a macromolecule or biologically degradable particle such that the additional component does not, to any appreciable extent, escape from the vascular compartment. The macromolecule may be optionally galactosylated.

Macromolecules to limit clearing component escaping from the vascular compartment are likely to be in excess of 500,000 Daltons and include carbohydrates such as dextrans, lipids as in liposomes or proteins such as in albumin microspheres or a macroglobulin. An example of a biodegradable particle for this purpose is an erythrocyte of blood group 0.

As an alternative to basing the first component on an antibody it may be based on a hormone or growth factor or substance other than an antibody and for which receptors capable of binding that substance exist on a tumour.

Tumours may express receptors for growth factor hormones and other metabolites such that these can be used as target sites for selective delivery. The corresponding growth factor, hormone, metabolite or genetic construct might then be used as the vector to carry an enzyme to

tumour sites in a comparable fashion to antibody. There are literature examples of radiolabelled hormones, growth factors and metabolites localising in tumours (Krenning et al, Lancet i 242-244, 1989 (Somatostatin); Hattner et al,
5 Am. J. Roentgenol. 143: 373-374, 1984) but in none of these were the vectors used to convey enzyme to tumour sites.

The enzyme part of the first component can be of human or non-human origin. The advantage of using an
10 enzyme of human origin lies in avoiding or minimising the immunogenic effect of an enzyme of non-human origin. The disadvantage of an enzyme of human origin is the probability that the presence of enzyme in human tissues will activate the prodrug, thus releasing active drug at
15 the non-tumour sites. However, it may be possible to identify certain human enzymes which are so distributed that this activation would not cause a serious problem. Also, inactivation of such enzymes in tissues might be achieved by using high affinity anti-enzyme antibody
20 fragments which would be rapidly cleared from the plasma before giving the first component conjugate. Where the human enzyme is normally present in the plasma, this would activate prodrug in the plasma which would be highly disadvantageous and be liable to cause general toxic
25 effects. Administration of an appropriately selected

antibody or antibodies or fragments directed at the enzyme in the conjugate would however also have the effect of inactivating free, naturally recurring enzyme of the same type in the plasma. In the case of human phosphatases
5 there are several different forms produced in different tissues but there is little evidence of specificity for substrates. There is also evidence that antibodies directed at one isotype of alkaline phosphatase may bind to other isotypes.

10 The immunogenicity of an enzyme of non-human origin may be reduced by modification of its amino acid sequence.

 In order to render the antibody-enzyme conjugate less immunogenic, it can be modified by conjugation to
15 polyethylene glycol or other polymers, e.g. by reaction with the cyanuric chloride derivative of methoxypolyethylene-glycol 5000. The resulting material may be employed directly, or may be pre-injected to render the host tolerant to further injections of the native
20 conjugate. Reaction with synthetic copolymers of D-glutamine acid and d-lysine or with tri-peptidyl-modified organic polymers comprising alternate D-glutamic acid and D-lysine on the exterior ends of the side chains can be predicted to depress the
25 immunogenicity of the conjugate. See, for example,

Abuchowsky A., van Es T., Palezuk NC, Davis FF - J. Biol. Chem. 252: (11), 3578-81, 1977, or
Kawamura K, Igarishji, T, Fujii T., Kamasaki J., Wada, H., Kishimoto, S. Int. Arch. Allergy appl. Immunol. 76:
5 324-330, 1985.

To minimise clinical problems arising from the use of immunogenic antibody enzyme conjugates and immunogenic antibodies or avidin-like constructs, it is desirable to minimise or delay the production of host
10 antibodies to xenospecific proteins by using immunosuppressive agents such as cyclosporin, cyclophosphamide, methotrexate, azathioprine etc., in order to provide sufficient time for the delivery of repeated treatments.

15 The ability of cyclosporin to prevent antimurine antibody responses by rabbits and in patients has been demonstrated. See, for example, Ledermann, JA. Begent, RHJ. Bagshawe, KD. Br. J. Cancer, 58: 562-566, 1988, or Ledermann, JA. Begent, RHJ. Riggs, SJ. Searle, F. Glaser,
20 MG, Green, AJ. Dale, RG. Br. J. Cancer 58: 654-657, 1988.

In certain clinical conditions, it can be advantageous for the first component to be conjugated to a signal producing molecule such as a radioisotope suitable for scintigraphic imaging by gamma camera so as to confirm

localisation of the first component at tumour sites.

Radiolabelling can be achieved with ^{125}I or ^{131}I with standard methods either using chloramine T (Greenwood F, Hunter W, Glover JS, Biochem. J. 89: 114-123, 1963, Fraker PJ, Speck JC, Biochem. Biophys. Res. Comm. 80: 849-857, 1978), but other methods of iodination or radiolabelling with other isotopes such as indium or technetium can also be used. Such radiolabelled conjugates are generally used in clinical practice in amounts required for radioimmunolocalisation by immunoscintigraphy and would generally form only a small part of the administered conjugate.

Modern methods of analysis may be used in conjunction with a radiolabelled fraction of the conjugate to determine the concentration of the conjugate at target sites and non-target sites and thus help determine the optimum time for administration of the prodrug. (Riggs et al, Int. J. Cancer Supp. 2, 95-98, 1988, Dewhurst et al, (Abstract) Br. J. Cancer 1988).

The system of the present invention can include more than one type of first component and/or more than one type of second component and/or more than one type of third component.

Heterogeneity in expression of target antigens and receptors on cells in tumours may require the use of more

than one vector to carry enzyme to tumour sites. Multiple vectors may permit greater or more economical delivery of enzyme to tumour sites. It may also be advantageous to use more than one type of prodrug to generate a state
5 equivalent to multidrug chemotherapy so as to reduce the risks of drug resistance and this in turn may require the use in treatment of more than one type of enzyme. These variations may in time require the use of more than one second component to achieve the required clearing of
10 enzymes from plasma and other non-tumour sites.

The three components forming the system of the present invention are designed to be used in association with one another in a method of treatment of the human or animal body by therapy.

15 It is specifically designed for use in a method for the treatment of malignant diseases including carcinomas, sarcomas, lymphomas and leukaemias which comprises administering to a host in need of such treatment an effective amount of a system.

20 In such a method, the first component is administered first, the second component is administered subsequent to the first component after a time interval such that the first component has selectively localised at the site of malignant cells and the third component is
25 administered subsequent to the second component after a

time interval such that the concentration of the first component in the blood has reduced from its peak value.

The following Examples are given to illustrate various aspects of the invention.

EXAMPLE 1

This is to illustrate the inactivation of an active enzyme site by an antibody.

A monoclonal antibody (SB43) was produced by conventional methods following immunisation of the lymphocyte donor mouse with carboxypeptidase G₂. Microtitre plates were coated with three units per well of carboxypeptidase G₂ and incubated with supernatants from the hybridoma culture, and it was found that ¹²⁵Iodine labelled rabbit anti-mouse antibody bound to the coated wells with a 50% binding titre at a dilution of the supernatant of 1:800 in buffered solution. Assay of enzyme activity was assessed after 24 hours incubation at 37°C in buffer containing a 1000-fold dilution of the antibody (hybridoma supernatant). Enzyme incubated with buffer alone for 24 hours retained most of its capacity to cleave methotrexate as shown by optical density measurements (54.1 carboxypeptidase units/ml initially

falling to 40 units/ml activity after 24 hours). In the wells containing the antibody (hybridoma supernatant) the activity was reduced to 13.0 carboxypeptidase units/ml. The antibody alone had no effect on the optical density of
5 methotrexate. These experiments show that the enzyme active site on the carboxypeptidase can be substantially inactivated by an antibody raised against the enzyme. Monoclonal antibodies to carboxypeptidase G₂, raised by the technique described above will only have a similar
10 enzyme inhibiting property if they are directed at epitopes in or close to the active site of the enzyme.

EXAMPLE 2

Evidence for localisation of antibody-enzyme conjugate at tumour sites

1. 4 nude mice bearing LS174T human colon cancer
5 xenografts on their L flanks were injected with A5B7 (Fab')₂ monoclonal antibody directed at carcinoembryonic antigen conjugated to carboxypeptidase G2 and labelled with ¹²⁵I. An immunoscintigraph taken after 48 hours confirms localisation of the conjugate at the tumour
10 sites.

Similar results were obtained using the following conjugates:

2. A5B7 intact IgG - carboxypeptidase
3. A5B7-F(ab')₂ - nitroreductase
4. SB10 (antiHCG) -F(ab')₂ - carboxypeptidase

2. METHODS OF CONJUGATION OF ANTIBODY TO ENZYME

5 Conjugation of IgG or F(ab')₂ with carboxypeptidase was accomplished by mixing a maleimide derivative of the enzyme with a thiolated antibody.

- 1) Thiolation with S-acetylthioglycolic acid N-hydroxysuccinimide ester (SATA).

10 IgG or F(ab')₂ in 0.1M sodium phosphate buffer, pH 7.6, (containing 372 mg of EDTA/litre) at 1-2 mg/ml was treated with a 15 molar excess of SATA (made up 20 mg/ml in DMF) and left at about 20°C for approximately 2 hours. The thiolated antibody was then passed down a column of
15 Sephadex G-25 to remove excess SATA. The thiol was deacetylated by adding 0.1 volumes of 3.5% hydroxylamine, pH 7.5, prepared by adding disodium hydrogen phosphate to an aqueous solution of hydroxylamine hydrochloride.

- 2) Thiolation with N-succinimidyl
20 3-(2-pyridyldithio)propionate (SPDP).

IgG or F(ab')₂ in 0.2M sodium phosphate buffer, pH 8.6, at a concentration of 4 mg/ml was treated with a 15 molar excess of SPDP in ethanol and left at r.t. for 1 hour. Excess SPDP was removed on a column of Sephadex G-25 equilibrated in 0.1M sodium acetate buffer, pH 4.5. The pyridyldisulphide group was then reduced for 30 minutes with 50 ul/ml of 100 mM dithiothreitol and excess reducing agent removed by Sephadex G-25 gel filtration.

3) Derivatisation of carboxypeptidase.

Carboxypeptidase in 0.1M sodium phosphate buffer, pH 7.6 (containing 372 mg EDTA/litre) at 2 mg/2.5 ml was treated for 3 hours with a 15 molar excess of succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB) dissolved in THF. The excess SMPB was removed by gel filtration on Sephadex G-25.

4) Conjugation

The derivatised enzyme was mixed with an equimolar amount of thiolated antibody and the progress of the conjugation monitored by gel filtration. When no further reaction was judged to take place the mixture was concentrated and the conjugate purified by gel filtration.

Typical enzyme activities obtained were 150-200 units/mg of conjugate.

EXAMPLE 3

Evidence that SB43 binds to carboxypeptidase G2 in vitro.

- 5 Microtitre wells were coated with
carboxypeptidase G2 and monoclonal antibodies SB43 (raised
to carboxypeptidase G2) and SB10 (raised to human
chorionic gonadotrophin) were added in dilution and
10 incubated before aspiration. ¹²⁵I anti-mouse IgG was
then added and incubated for 30 minutes followed by
aspiration and washing. The wells were cut out and
counted in a gamma counter. The results set out below
show no significant binding of SB10 to the
15 carboxypeptidase coated wells but all dilutions of SB43
used showed high counts indicating binding to the
carboxypeptidase. SB43 modified by addition of galactose
moieties was included a similar dilution and showed
similar binding to unmodified SB43.
- 20 The microtitre plate was coated with 0.1 microg.
CPG₂ per well and incubated overnight with SB43. The
¹²⁵I-mouse IgG was then introduced, the plate incubated
for 1 hour, washed and radio-counted.

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| | SAMPLE | TIME | COUNTS (1) | CPM (1) | %CV | |
|----|--------|------|------------|---------|-----|----------------------------------|
| | 1 | 30 | 1092 | 2171.4 | 3.1 | (Negative Control-SB10-Anti-hCG) |
| | 2 | 30 | 805 | 1588.6 | 3.6 | " |
| | 3 | 30 | 16428 | 33278.0 | .8 | SB43 x 20 |
| 5 | 4 | 30 | 23339 | 47302.9 | .7 | " |
| | 5 | 30 | 22020 | 44679.3 | .7 | SB43 x 100 |
| | 6 | 30 | 22096 | 44833.8 | .7 | " |
| | 7 | 30 | 8437 | 16873.4 | 1.1 | SB43 x 1000 |
| | 8 | 30 | 7671 | 15336.8 | 1.1 | " |
| 10 | 9 | 30 | 15411 | 31052.2 | .8 | SB43-Gal-10x20 |
| | 10 | 30 | 15418 | 31066.3 | .8 | " |

EXAMPLE 4Evidence that SB43 inactivates/clears Ab-E-conjugate in vivo from plasma

The level of carboxypeptidase G2 activity in plasma can be monitored by observing the hydrolytic cleavage of methotrexate, a folic acid analogue, to pterates and L-glutamate. When a conjugate of A5B7-F(ab')₂-carboxypeptidase G2 (25 enzyme units) was injected intravenously and plasma samples obtained 20 hours later significant hydrolysis of methotrexate was observed equivalent to 1.12 to 1.45 enzyme units/ml as shown by the steps of the spectrophotometric print-out.

Mice which were injected with galactosylated anti-carboxypeptidase (SB43-Gal 10) 19 hours after A5B7F(ab')₂-CPG2 and plasma taken 5 minutes and 15 minutes later caused no significant hydrolysis of methotrexate showing that the enzyme had been inactivated and/or cleared from the plasma.

EXAMPLE 5

Biotinylation of antibody-enzyme conjugate

Carboxypeptidase G2 (44.4 mg) in 0.05M sodium bicarbonate buffer, pH 8.5 (1.5 ml) was mixed with sulphosuccinimidyl 1-6-(biotinamide) hexanoate (292 ug in 73 ul of buffer and left at room temperature for 3 hours. The enzyme was then separated on Sephadex G-25 equilibrated in 0.15M sodium phosphate buffer pH 7.6, containing 372 mg EDTA/litre and the volume adjusted to 2.5 ml. The biotinylated enzyme was then treated for 3 hours with a 15 molar excess of succinimidyl 4 - (p-maleimido phenyl)butyrate (SMPB) dissolved in tetrahydrofuran and the excess SMPB removed by gel filtration on Sephadex G25. The derivatised enzyme was then conjugated to thiolated F(ab')₂ fragment of the A5B7 antibody as described in Example 2.

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Affinity purified avidin was used as obtained from Sigma Ltd., 10-15 units/mg protein. Mice received 20 ug of biotinylated A5B7-carboxypeptidase G2 conjugate followed after one hour by avidin in the dose range
5 20-500 ug. Rapid clearance of the enzyme activity in plasma was observed comparable to that observed with SB43 monoclonal antibody in Example 4.

EXAMPLE 6

IgG class immunoglobulins carrying different
10 specificities on their two binding sites can be made by a fusion technique employing hybridomas producing different antibodies (Milstein C & Cuello AC. Nature 305: 537-540, 1983; Sfaerz UD & Bevan MJ. Proc. Nat. Acad. Sci. USA 83: 1453-1457, 1986) or by chemical conjugation of univalent
15 preparations of each of the antibodies required as used here. F(ab')₂ fragments of monoclonals SB10 (anti-human chorionic gonadotrophin (anti-hCG)) and A5B7 (anti-carcinoembryonic antigen, (anti-CEA)) were reduced in the presence of arsenite. F(ab')₂ fragment (20 mg) in
20 0.1M sodium phosphate buffer pH 7.6 (10 ml) was mixed with sodium arsenite (12.4 mg) EDTA (3.72 mg) and 2-mercapto-ethylamine (1.13 mg) and left at room temperature. Solid 5,5'-dithio-bis-(2-nitrobenzoic acid)

(19.8 mg) was added and the mixture left at about 20°C for 18 hours. The thionitrobenzoate modified Fab' (TNB derivative) was purified by gel filtration on Sephadex G-25 with a yield of approximately 70% based on protein recovery.

The TNB derivative of anti-CEA (4.8 mg) in 5 ml of 0.1M sodium phosphate buffer, pH 6.8, containing 1mM EDTA was treated for 30 minutes with mercaptoethylamine to give a final concentration of 10mM. The reduced TNB-anti-CEA Fab' was then purified by gel filtration on Sephadex G-25 equilibrated in 0.1M sodium phosphate buffer pH 7.0, containing 1mM EDTA. The reduced TNB-anti-CEA Fab' was then incubated with 4.9 mg (5 ml) of TNB derivative of anti-hCG for 16 hours and the formation of bispecific antibody monitored by gel filtration on a Superose S-12 column (Pharmacia). The yield was 20% based on protein after purification of the bispecific antibody on the Superose S-12 column. The ability of this ¹²⁵I labelled biospecific antibody to function in vivo and bind to its corresponding antigen was demonstrated by injection into nude mice bearing either CEA producing LS174T tumours or hCG producing CC3 tumours. At 20 hours post-injection mean tumour to organ ratios were:

| | anti CEA/ anti hCG | non-specific F(ab') ₂ |
|---|--------------------|----------------------------------|
| | blood 2.9 | 0.6 |
| | liver 3.9 | 1.9 |
| | kidney 1.8 | 1.2 |
| 5 | lung 3.2 | 1.2 |
| | spleen 6 | 3.0 |
| | colon 9 | 5.3 |

(Conjugation of A5B7 and SB43 (anticarboxypeptidase) has not yet been performed but above experiment demonstrates
10 retention of binding site function).

EXAMPLE 7

Method for galactosylation

Cyanomethyl 2,3,4,6-tetra-O-acetyl-1-thio- β -D) galactopyranoside (400 mg) in anhydrous methanol (10 ml)
15 was treated with 5.4 mg of sodium methoxide in 1 ml of anhydrous methanol at about 20°C for 48 hours. A stock solution of IgG in 0.25M sodium borate buffer, pH 8.5 at 1.3 mg/ml was prepared. Since the number of galactose residues conjugated to IgG was not determined, a unitage
20 was adopted corresponding to the number of microlitres of the activated galactose derivative added to 200 μ g of IgG at a concentration of 1.3 mg/ml.

Aliquots of the activated galactose derivative (e.g. 300, 80, 40, 10, 5 and 2 μ l) were dispensed into 3 ml glass ampoules and evaporated to a glassy residue in a stream of nitrogen under vacuum. 200 micrograms of IgG (153 μ l of stock solution) were added to each aliquot and mixed until the residue was dissolved. After 2 hours at about 20°C the solution was dialysed against 3 changes of PBS (phosphate buffer saline). Tests were performed to determine what level of galactosylation gave the most effective results. It was found that, in terms of the unitage defined above, 10 μ l of the activated galactose derivative added to 200 μ g of IgG gave the most satisfactory results. Figure 1 shows blood and tumour levels up to 48 hours after injection of monoclonal SB43 (anti-carboxypeptidase). A, native form; B, galactosylated form. Further studies were performed, each in groups of 4 mice, bearing LS174T tumours and receiving A5B7.F(ab')₂-CP (carboxypeptidase G2) conjugate followed after 24 hours by SB43 (anti-carboxypeptidase) galactosylated to the 10 μ l level (as defined previously) or with saline as control followed one hour later by the bis-chloro benzoic acid mustard prodrug, 4-[bis-(2-chloroethyl)amino]-benzoic acid glutamide. Mice were killed at intervals following administration of the prodrug, the tissues extracted and prodrug and active drug levels were measured by HPLC methods.

Figure 1B shows the levels of active drug in various tissues in mice which had not received SB43-Gal 10 clearing antibody with those that had. In the absence of SB43-Gal 10 clearing antibody, levels of active drug were significantly lower than those found in liver and lung but in animals receiving the SB43-Gal 10 clearing antibody tumour levels were higher than in any other tissue.

As part of the same experiment two groups of mice, one with and one without SB43-Gal 10, were killed without receiving the prodrug. The tissues were extracted and tested for ability to convert prodrug to active drug in vitro. The results are shown in the Table and expressed as percentage of injected dose of carboxypeptidase per gram of tissue.

15 In vivo admin Carboxypeptidase G2% i.v. dose per gram of tissue at 48 hours

| | Tumour | Plasma | T/P |
|-----------------|------------|--------|-----|
| Ab-CPG2 | 8.1(+0.69) | 0.22 | 36 |
| AB-CPG2 | 7.2(+1.42) | 0.026 | 277 |
| + | | | |
| Gal 10 antiCPG2 | | | |
| 24 hours later | | | |

EXAMPLE 8

Where the antigen corresponding to an intravenously administered antibody is present in the blood, antigen-antibody complexes form and these accelerate clearance of the antibody from the circulation into the reticuloendothelial cells. Accelerated clearance of anti-hCG antibodies W14 and SB10 occurs when these are injected into nude mice bearing CC3 hCG secreting tumours when compared with A5B7 anti-CEA antibody in LS174T bearing mice which express CEA on LS174T cell membranes but do not secrete CEA into the blood.

Figure 2 shows the result of administering SB10 $F(ab')_2$ -CP (50 units CP) intravenously to 6 CC3 bearing nude mice, followed by the first of three injections (10 mg each) of the monomesyl monochloro benzoic acid mustard prodrug 4-[(2-chloroethyl)mesylamino]benzoic acid glutamide, the second given at 56 hours and the third at 72 hours. After 2 weeks the tumour was no longer detectable and the mice remain tumour free at 12 weeks. The growth of CC3 tumours in 6 untreated mice is also shown.

Attempts to introduce the prodrug into LS174T bearing mice before 120 hours after administration of A5B7

F(ab')₂-CP 50 units resulted in death of the animals and this was shown to be due to persisting enzyme activity in the blood.

Figure 2b shows accelerated clearance of 20 ug monoclonal ¹²⁵I-SB43 anticarboxypeptidase from the blood of A2G mice when 77 ug of the corresponding antigen, carboxypeptidase G2, was administered 1 hour later compared with controls which did not receive the antigen.

These data indicate that accelerated clearance of an administered antibody can be achieved by administration of a substance expressing the epitope corresponding to the binding site of the antibody.

EXAMPLE 9

Conjugation of TCK9 human albumin microspheres to SB43

1 mg of TCK9 human polyalbumin microspheres were derivatised with a 12.5M excess of sulpho-MBS (based on monomeric unit of 66 Kd) in a total of 1 ml phosphate buffer pH 7.8 for 2 hours at about 20°C. The mixture was centrifuged at 3000 rpm for 3 minutes and resuspended in 1 ml of buffer, and rewashed once more. 1.5 mg of ¹²⁵I labelled SB43 was thiolated by 20M excess of SPDP, according to manufacturers (Pharmacia) instructions, at

about 20°C. The derivatised microspheres were centrifuged at 3000 rpm for 3 minutes, and then resuspended in the thiolated SB43 solution, the conjugation was carried out by incubating the mixture at 5 4°C for 72 hours.

The antibody polyalbumin conjugate was separated from the reaction mixture by centrifuging at 13000 rpm (MSE Micro centaur) for 2 minutes; the pellet was resuspended in 250 ul of sterile saline for use.

EXAMPLE 10

Asialofetuin was given intravenously at time zero and again at 120 minutes to mice bearing LS174T xenografts. ¹²⁵I-A5B7-galactosylated to 10 units was administered at time +5 minutes. Mice were killed at 15 intervals and tissues excised and radioactivity levels counted. At 24 hours the tumour to blood ratio was 27.8:1 and the tumour to liver ratio 4.2:1. All other tissues showed even more favourable ratios. It should be recognised that whereas enzyme taken up by liver is 20 rapidly inactivated, radioactivity persists in the organ.

CLAIMS

1. A three component system, for use in association with one another, in the treatment of malignant diseases comprising: a first component which is a substance or conjugation of substances characterised by (a) one or more
5 molecular configurations that are complementary to molecular configurations associated with malignant cells, such that the first component tends to localise selectively at sites of malignant cells and (b) additionally by one or more catalytic sites; a second
10 component which is able to bind to such part of the first component so as to inactivate the catalytic site of the first component and/or accelerate the clearance of the first component from the blood when the first and second components are administered clinically and a third
15 component which is a substrate for the catalytic site on the first component, one of the end products of the catalysis being a substance which is more cytotoxic to malignant cells than the third component.
2. A system according to claim 1 wherein the first
20 component or a part of the first component is biotinylated and in which the second component is avidin or other protein with a high affinity for binding to biotinylated proteins.
3. A system according to claim 1 wherein the first
25 component is a conjugate of an antibody to a tumour associated antigen or a fragment thereof that includes the antigen binding site of the antibody, said antibody or

fragment thereby being conjugated directly, or indirectly through a linking component to an enzyme or to an antibody or antibody fragment with catalytic functions.

4. A system according to claim 3 wherein the
5 conjugation is effected by chemical bonding or by splicing together nucleic acid sequences that code at least for one or more antigen binding sites and one or more catalytic sites and such other sequences as are necessary to retain the vector function of the molecule and the catalytic
10 function of the peptide when the gene product of the reconstructed nucleic acid sequence is expressed by eukaryotic or prokaryotic cells.

5. A system according to claims 3 or 4 wherein the antibody in the first component is bivalent and formed by
15 bonding together two univalent antibody fragments, or by recombinant DNA techniques, one fragment having affinity for a tumour marker substance, the other having affinity for an enzyme.

6. A system according to claim 5 wherein the
20 conjugate between antibody and enzyme is formed either in vitro prior to administration or in vivo by first administering the bivalent antibody, allowing time for it to localise at tumour sites and then administering the enzyme for capture by the second arm of the antibody
25 localised at tumour sites.

7. A system according to any one of the preceding claims in which the antibody of the first component is a

human immunoglobulin, or fragment thereof, having antigen binding site(s) of human origin or having antigen binding site(s) of non-human species.

8. A system according to any one of the preceding
5 claims wherein the second component is an antibody or fragment thereof having an affinity for an antigen binding site of an antibody of the first component or the active site of an enzyme of the first component or another constituent part of the first component.
- 10 9. A system according to any one of the preceding claims wherein the second component is one causing rapid loss of enzyme activity of the first component in plasma without incurring significant loss of enzyme activity from tumour sites.
- 15 10. A system according to claim 9 wherein the second component includes a sufficient number of covalently bound galactose residues or residues of other sugars such as lactose or mannose, so that it can bind enzyme in plasma but be removed together with the enzyme or antibody-enzyme
20 conjugate from plasma by receptors for galactose or other sugars in the liver in a period of time such that the antibody does not, to any appreciable extent, enter the extravascular space of the tumour where it could inactivate tumour localised enzyme.
- 25 11. A system according to claim 10 wherein galactose residues in the second component are exposed by removing terminal sialic acid residues.

12. A system according to claim 1 wherein the first component is an antibody enzyme conjugate modified by addition of, or exposure of, additional galactose or other sugar residues, and is for administration in conjunction
5 with an agent, such as asialofetuin, which has greater affinity for the corresponding sugar receptors which are involved in removing galactosylated or similar proteins from the blood, the asialofetuin blockade being maintained until satisfactory levels of conjugate have been attained
10 in the tumour, then allowing the concentration of the conjugate in the plasma to fall before administering the third component.
13. A system according to any one of claims 1 to 11 wherein the second component is conjugated to a
15 macromolecule such as a dextran, liposome, albumin microsphere or macroglobulin with a molecular weight in excess of 500,000 Daltons or a biodegradable particle such as a blood group 0 erythrocyte so that the second component is restrained from leaving the vascular
20 compartment by virtue of the size of the conjugate.
14. A system according to any one of the preceding claims wherein the second component is an antigen, hapten or protein construct bearing an epitope capable of binding with the first component to form complexes having
25 accelerated clearance from plasma.
15. A system according to claim 1 or 2 wherein the first component includes a hormone or growth factor or substance other than an antibody and for which receptors capable of binding that substance exist on a tumour.

16. A system according to any one of the preceding claims wherein the first component includes an enzyme of human or non-human origin.
17. A system according to claim 16 wherein the enzyme is of non-human origin but modified by amino acid substitutions to minimise its immunogenicity in man.
18. A system according to claim 17 wherein the enzyme is conjugated to residues such as polyethylene glycol, or other polymers to reduce immunogenicity.
19. A system according to any one of the preceding claims wherein the first component is conjugated to a signal producing molecule such as a radioisotope suitable for scintigraphic imaging by gamma camera so as to confirm localisation of the first component at tumour sites.
20. A system according to any one of the preceding claims including more than one type of first component and/or more than one type of second component and/or more than one type of third component.
21. A system according to any one of the preceding claims for use in association with cyclosporin or other immunosuppressive drug or antibody to inhibit antibody response in the host to the system.
22. A system according to any one of the preceding claims for use in a method of treatment of one human or animal body by therapy.
23. A method for the treatment of malignant disease including carcinomas, sarcomas, lymphomas and leukaemias

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which comprises administering to a host in need of such treatment an effective amount of a system according to any one of claims 1 to 21.

24. A method according to claim 23 wherein the first
5 component is administered first, the second component is administered subsequent to the first component after a time interval such that the first component has selectively localised at the site of malignant cells and the third component is administered subsequent to the
10 second component after a time interval such that the concentration of the first component in the blood has reduced from its peak value.

Fig. 1A.

1/2

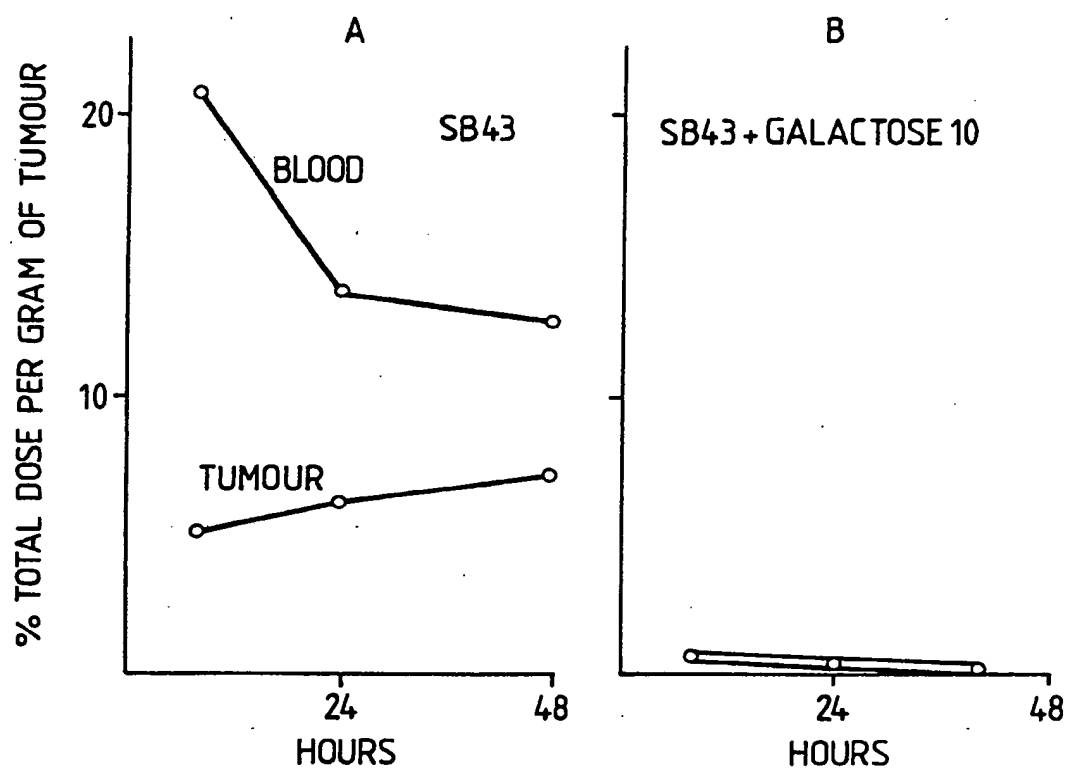
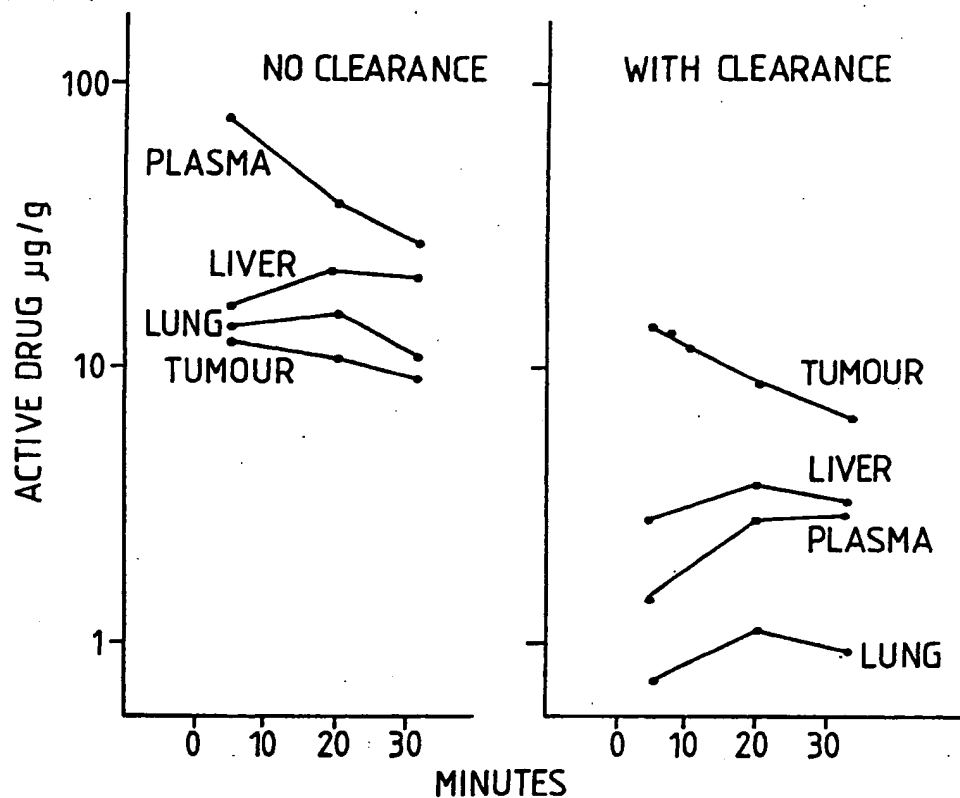


Fig. 1B.



ACTIVE DRUG MEASURED (HPLC) AS CONVERSION OF PRODRUG BY TISSUE EXTRACT AT INTERVALS FOLLOWING SB43 GAL 10 CLEARING A/B.

2/2

Fig. 2A.

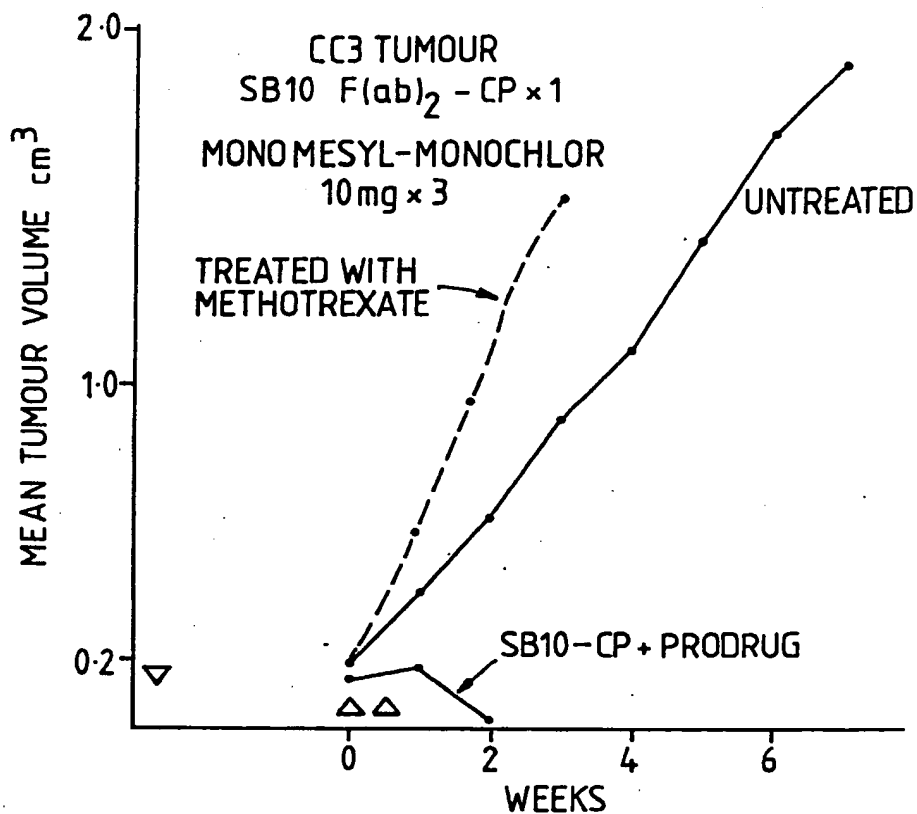
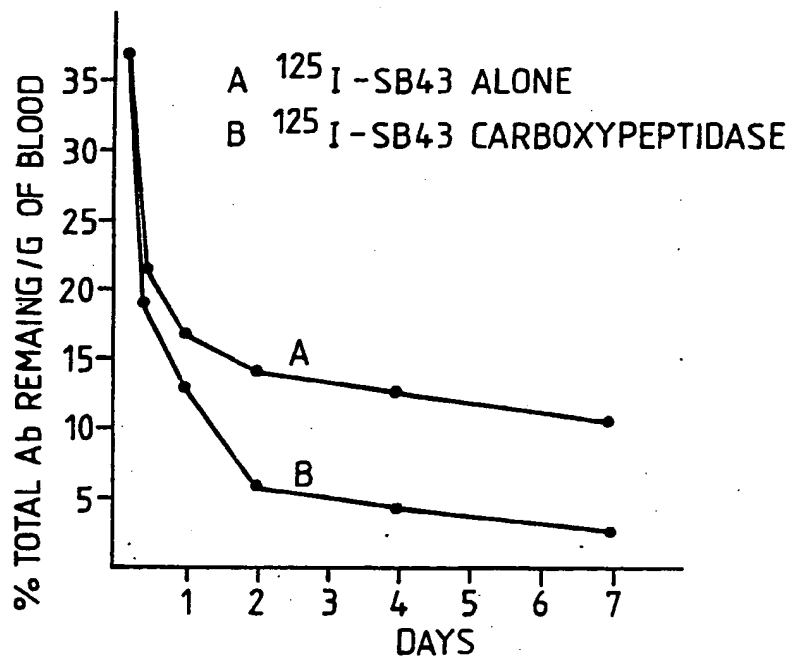


Fig. 2B.



SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 89/00427

| I. CLASSIFICATION F SUBJECT MATTER (If several classification symbols apply, indicate all) ⁴ According to International Patent Classification (IPC) or to both National Classification and IPC IPC ⁴ : A 61 K 39/395, A 61 K 39/44, A 61 K 45/05 | | | | | | | | | | | | | | | | | |
|--|---|-------------------------------------|--|--|--|---|--|------|---|---|------|---|---|------|-----|--|------|
| II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Minimum Documentation Searched ⁷</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 25%; border-bottom: 1px solid black;">Classification System</td> <td style="border-bottom: 1px solid black;">Classification Symbols</td> </tr> <tr> <td style="padding: 10px;">IPC⁴</td> <td style="padding: 10px;">A 61 K</td> </tr> </table> <div style="border-top: 1px solid black; padding-top: 10px;"> Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸ </div> | | | Classification System | Classification Symbols | IPC ⁴ | A 61 K | | | | | | | | | | | |
| Classification System | Classification Symbols | | | | | | | | | | | | | | | | |
| IPC ⁴ | A 61 K | | | | | | | | | | | | | | | | |
| III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border-bottom: 1px solid black;">Category ⁹</th> <th style="width: 60%; border-bottom: 1px solid black;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 30%; border-bottom: 1px solid black;">Relevant to Claim No. ¹³</th> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;">EP, A, 0140728 (SANOFI) 8 May 1985 --</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-22</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;">EP, A, 0186551 (SANOFI) 2 July 1986 --</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-22</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;">EP, A, 0089880 (SANOFI) 28 September 1983 --</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-22</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">A,P</td> <td style="padding: 5px;">WO, A, 88/07378 (CANCER RESEARCH CAMPAIGN TECHNOLOGY LTD) 6 October 1988 -----</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-22</td> </tr> </table> | | | Category ⁹ | Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹² | Relevant to Claim No. ¹³ | A | EP, A, 0140728 (SANOFI) 8 May 1985 -- | 1-22 | A | EP, A, 0186551 (SANOFI) 2 July 1986 -- | 1-22 | A | EP, A, 0089880 (SANOFI) 28 September 1983 -- | 1-22 | A,P | WO, A, 88/07378 (CANCER RESEARCH CAMPAIGN TECHNOLOGY LTD) 6 October 1988 ----- | 1-22 |
| Category ⁹ | Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹² | Relevant to Claim No. ¹³ | | | | | | | | | | | | | | | |
| A | EP, A, 0140728 (SANOFI) 8 May 1985 -- | 1-22 | | | | | | | | | | | | | | | |
| A | EP, A, 0186551 (SANOFI) 2 July 1986 -- | 1-22 | | | | | | | | | | | | | | | |
| A | EP, A, 0089880 (SANOFI) 28 September 1983 -- | 1-22 | | | | | | | | | | | | | | | |
| A,P | WO, A, 88/07378 (CANCER RESEARCH CAMPAIGN TECHNOLOGY LTD) 6 October 1988 ----- | 1-22 | | | | | | | | | | | | | | | |
| <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div> | | | | | | | | | | | | | | | | | |
| IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;"> Date of the Actual Completion of the International Search <div style="text-align: center; padding-top: 5px;">18th August 1989</div> </td> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;"> Date of Mailing of this International Search Report <div style="text-align: center; padding-top: 5px;">22.09.89</div> </td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 5px;"> International Searching Authority <div style="text-align: center; padding-top: 5px;">EUROPEAN PATENT OFFICE</div> </td> <td style="border-bottom: 1px solid black; padding: 5px;"> Signature of Authorized Officer <div style="text-align: center; padding-top: 10px;"> L. ROSSI </div> </td> </tr> </table> | | | Date of the Actual Completion of the International Search <div style="text-align: center; padding-top: 5px;">18th August 1989</div> | Date of Mailing of this International Search Report <div style="text-align: center; padding-top: 5px;">22.09.89</div> | International Searching Authority <div style="text-align: center; padding-top: 5px;">EUROPEAN PATENT OFFICE</div> | Signature of Authorized Officer <div style="text-align: center; padding-top: 10px;"> L. ROSSI </div> | | | | | | | | | | | |
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| International Searching Authority <div style="text-align: center; padding-top: 5px;">EUROPEAN PATENT OFFICE</div> | Signature of Authorized Officer <div style="text-align: center; padding-top: 10px;"> L. ROSSI </div> | | | | | | | | | | | | | | | | |

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers 2, because they relate to subject matter not required to be searched by this Authority, namely:

2) claims 23,24 See PCT Rule 39.1.iv Methods for treatment of the human or animal body by surgery or therapy as well as diagnostic methods.

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers _____, because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This international Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the international Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

GB 8900427
SA 28296

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 15/09/89
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
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| | | JP-A- 61027926 | 07-02-86 |
| EP-A- 0186551 | 02-07-86 | FR-A, B 2573656 | 30-05-86 |
| | | AU-A- 5047385 | 05-06-86 |
| | | CA-A- 1254139 | 16-05-89 |
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| | | US-A- 4749566 | 07-06-88 |
| EP-A- 0089880 | 28-09-83 | FR-A, B 2523445 | 23-09-83 |
| | | AU-B- 563356 | 09-07-87 |
| | | AU-A- 1250483 | 22-09-83 |
| | | JP-A- 58208238 | 03-12-83 |
| | | OA-A- 7388 | 30-11-84 |
| | | US-A- 4762707 | 09-08-88 |
| WO-A- 8807378 | 06-10-88 | None | |